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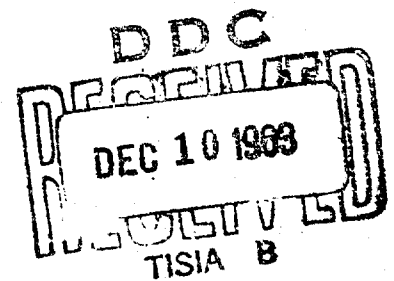
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TECHNICAL MANUSCRIPT 95

FACTORS AFFECTING PLAQUE FORMATION BY THE INFECTIOUS RIBONUCLEIC ACID OF THE EQUINE ENCEPHALITIS VIRUSES

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FACTORS AFFECTING PLAQUE FORMATION BY THE INFECTIOUS
RIBONUCLEIC ACID OF THE EQUINE ENCEPHALITIS VIRUSES

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ABSTRACT

Factors that influence plaque formation by the infectious RNA of the equine encephalitis viruses were studied. Optimal conditions were found that provided a very sensitive and reliable assay system for the infectious RNA of this group of viruses. There was a linear relationship between plaque counts in monolayers and the dilution of the RNA applied. The frequency distribution of plaques formed by infectious RNA is of the Poisson type, suggesting that one RNA molecule gives one plaque.

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I. INTRODUCTION

Successful isolation of infectious ribonucleic acid (RNA) by phenol extraction from a number of animal viruses, including Eastern equine encephalitis (EEE) virus,¹ Western equine encephalitis (WEE) virus,² and Venezuelan equine encephalitis (VEE) virus in this laboratory, prompted interest in methods for quantitative assay of the infectivity of the viral RNA. Mayer and Sokol³ described a quantitative plaque assay of the infectivity of RNA from EEE virus that was more sensitive than the system previously reported by Wecker.² They reported that the plaque titer of the infectious RNA was 0.001 to 0.01 per cent of the whole-virus titer of the original brain tissue homogenate used for the isolation of the RNA.

This report concerns investigation of the effects of numerous factors on plaque formation by infectious RNA of encephalitis viruses and the consequent development of a very sensitive and reproducible assay system.

II. MATERIALS AND METHODS

A. VIRUSES

EEE virus (C.D.C. strain SC#7), used in the majority of the experiments, was received as 20 per cent chick embryo suspension from the Communicable Disease Center, Montgomery, Alabama. It has been passaged two times in chick embryo in this laboratory. VEE virus (Trinidad strain), previously described in Hardy,⁴ and WEE virus (Rockefeller strain) obtained from the U.S. Army Medical School as a ten per cent suspension of fourth mouse brain passage, were also used in some of the experiments.

B. REAGENTS

1. Stock EDTA

One gram of disodium ethylenediaminetetraacetate (EDTA) was dissolved in 100 milliliters demineralized water. The solution was sterilized by autoclaving and stored frozen in the required proportion with the stock trypsin.

2. Stock Trypsin

One gram of trypsin (Difco, 1:250) was dissolved with 100 milliliters of sterile demineralized water and agitated with a magnetic stirrer for

30 minutes. The insoluble material was separated by centrifugation for 10 minutes at 360g. The supernatant fluid was sterilized by filtering through a Seitz filter with vacuum. Two milliliters of trypsin stock solution were combined with 1.5 milliliters of stock EDTA and stored frozen in screw-cap tubes.

3. Trypsin-EDTA Solution

The working trypsin-EDTA solution contained 1.5 milliliters of the stock EDTA solution and 2 milliliters of the stock trypsin solution in 100 milliliters of sterile saline A.⁵ This solution was adjusted to pH 8.8 to 8.9 with 0.7 milliliter of 5.6 per cent sodium bicarbonate.

C. PREPARATION OF INFECTIOUS RNA

Moribund embryos from eggs infected with EEE, VEE, or WEE viruses were homogenized at room temperature for one minute in a Waring blender with phenol* and phosphate buffer (9 milliliters of 83 per cent phenol and 4.5 milliliters of 0.02M phosphate buffer, pH 7.4, per embryo). The homogenate was centrifuged for 15 minutes at 17,300g in an angle-head, refrigerated centrifuge and the aqueous layer was then carefully removed. Second and third phenol extractions were carried out at room temperature by shaking the preparation in a glass-stoppered flask for five minutes, each time adding 83 per cent phenol solution in a quantity equal to the volume of the aqueous phase. Centrifugation and recovery of aqueous layers was performed as for the initial extraction. Before the third extraction, the volume of the aqueous layer was increased one-third by the addition of 0.02M phosphate buffer, pH 7.4. To precipitate the RNA, the aqueous phase from the final extraction was treated with 66 per cent cold ethanol in the presence of two per cent potassium acetate for one hour at -10°C. The precipitate of RNA was separated by centrifugation and resuspended in 0.02M phosphate buffer (one milliliter per embryo) and then reprecipitated with 1M NaCl at 0°C for two hours. Immediately after isolation, the RNA precipitate was dissolved in 0.02M phosphate buffer (two milliliters per embryo) and stored at -65°C. The infectivity of the RNA prepared under these conditions ranged from one to ten per cent of the infectivity shown by intact virus in untreated homogenates of similarly infected embryos prepared in 0.02M phosphate buffer (pH 7.4) or heart infusion broth (Table I). All RNA preparations were completely inactivated when treated with ribonuclease (one microgram per milliliter) for five minutes at 37°C.

* Phenol, C.P.: Special, Absolute, National Aniline Division, Allied Chemical Corporation, New York, New York.

TABLE I. INFECTIVITY OF RIBONUCLEIC ACID FROM EASTERN
AND VENEZUELAN EQUINE ENCEPHALITIS VIRUSES^{a/}

PREPARATION	VIRUS TITER, pfu/ml ^{b/}	RNA TITER, pfu/ml	RNA TITER x 100 VIRUS TITER
EEE	1.0×10^8	1.4×10^7	1.4
EEE	4.1×10^8	6.2×10^6	0.2
EEE	1.4×10^8	1.5×10^8	10.0
VEE	2.0×10^8	3.4×10^6	1.7
VEE	1.0×10^8	1.5×10^6	1.5

a. RNA was extracted from infected chick embryos by the method described in Section II.

b. pfu/ml = plaque-forming units per milliliter.

D. PREPARATION OF LACT-CALF GROWTH MEDIUM

Growth medium for monolayers consisted of 0.5 per cent lactalbumin hydrolyzate, 10 per cent calf serum, 0.075 per cent sodium bicarbonate, and 0.002 per cent phenol red in Hanks' balanced salt solution (BSS). The medium was sterilized by filtration through an asbestos pad in a Seitz apparatus.

E. PREPARATION OF CHICK FIBROBLAST MONOLAYERS FOR PLAQUE ASSAY

Minced tissues from eight 10-day-old chick embryos were trypsinized with agitation at 37°C in two steps. (a) Tissues were treated with 20 milliliters of the trypsin-EDTA solution for 10 minutes and the fluid portion was discarded. (b) These same tissues were then treated for an additional 25 minutes with 80 milliliters of fresh trypsin-EDTA solution. Three milliliters of sterile, normal calf serum were mixed with the trypsinized tissues and then the fluid portion was centrifuged in the cold at 250g for five minutes to collect the cells. The pellet of cells and tissue fragments was resuspended in 150 milliliters of lact-calf growth medium and filtered through four layers of sterile cheesecloth (grade 120). Cell counts were made on this filtered suspension and the required volume of lact-calf medium was added to give a cell density of 4.0×10^5 cells per milliliter. Antibiotics were added to give a final concentration of 250 micrograms of dihydrostreptomycin sulfate and 125 units of penicillin

G sodium per milliliter in the cell culture. Five-milliliter portions of this cell suspension were placed in 60-mm plastic Petri dishes and were incubated 24 to 48 hours at 37°C in an atmosphere of 95 per cent air and five per cent carbon dioxide before inoculation with appropriate virus or infectious RNA suspension.

F. PREPARATION OF NUTRIENT AGAR OVERLAY

The nutrient agar overlay was prepared in two solutions: Solution A consisted of 2.2 per cent agar; Solution B consisted of 1.0 per cent lactalbumin hydrolyzate, 1.0 per cent gelatin, and 0.2 per cent yeast extract. The solutions were all prepared in Hanks' BSS without phenol red indicator. Solutions A and B were autoclaved separately (15 minutes at 120°C). After sterilization, Solution A (agar) was held at 50°C and Solution B (nutrient) at 30°C. Antibiotics and sodium bicarbonate were added to the autoclaved and cooled Solution B to give the following concentrations in the complete overlay: penicillin, 125 units per milliliter; streptomycin, 250 micrograms per milliliter; sodium bicarbonate, 0.14 per cent. Equal volumes of the two solutions were mixed just before use as an overlay. This overlay was used in the assay of infectious RNA and also for the assay of whole virus.

G. ASSAY OF VIRUS INFECTIVITY

The tissue culture plaque method was used for the titration of intact virus. The virus, appropriately diluted in heart infusion broth, was pipetted in 0.1-milliliter volumes onto 24- or 48-hour monolayers that had been washed once with saline A. After 20 minutes' adsorption at 25°C, the excess inoculum was removed and the monolayers were overlaid with nutrient agar. Plates were incubated at 37°C in a humidified atmosphere with five per cent added carbon dioxide.

III. EXPERIMENTS AND RESULTS

A. BACKGROUND

When the study of infectious RNA from VEE, WEE, and EEE viruses began in our laboratory, the assay used for nucleic acid infectivity was a modification of the Koch *et al*⁸ tissue culture system for the assay of infectious RNA from poliovirus. Although the method sometimes gave satisfactory results, it often failed to yield plaques and agreement among replicate assays was unsatisfactory. Since the infectivity assay was fundamental to our investigations, we undertook the study of the effect of a number of factors on plaque formation by infectious RNA from EEE, WEE, and VEE viruses. Only data involving experiments with infectious RNA from EEE virus will be shown here, but the results and the discussions are applicable also to the RNA of VEE and WEE viruses.

B. FACTORS INFLUENCING PLAQUE FORMATION BY INFECTIOUS RNA

In the experiments to be described, the conditions were those found to be optimal for all factors other than the one under investigation. Unless otherwise indicated, in all tests cells were washed first with saline A, then with 0.5M NaCl, and finally with 1M NaCl-0.1M Tris, pH 8.2, and the RNA was diluted in the Tris-buffered 1M NaCl.

1. RNA Diluent

A comparison of different diluents (demineralized water, 0.02M phosphate buffer, 2.0M MgSO_4 , 1.125M MgSO_4 , and 1M NaCl buffered with Tris-(hydroxymethyl)aminomethane at pH 7.2 to 8.2) revealed that 1M NaCl-Tris at pH 8.2 insured the highest infectivity of RNA for chick embryo fibroblast monolayers (Table II).

TABLE II. PLAQUE FORMATION^{a/} BY EEE VIRUS RNA:
EFFECT OF DILUENT

DILUENT	DILUTION	PLAQUE COUNTS, ^{b/} per plate	INFECTIVITY pfu/ml
Demineralized water	10^{-3}	32,60,67,86	$10^{5.7}$
	10^{-4}	9,6,1,3	
	$10^{-4.7}$	0,1,2,0	
0.02M phosphate buffer, pH 7.4	10^{-3}	TM, TM, TM, TM ^{c/}	$10^{6.2}$
	10^{-4}	6,32,50,0	
	$10^{-4.7}$	4,1,2,5	
1.125M MgSO_4 , pH 7.9	$10^{-4.7}$	0,0,1,1,2	$10^{5.6}$
	10^{-5}	0,0,0,0,0	
1M NaCl, 0.1M Tris, pH 8.2	$10^{-4.7}$	50,82,51,57,67,59	$10^{7.5}$
	10^{-5}	35,26,21,32,36,50	

- a. The monolayers were washed with saline A, 0.5M NaCl, and 1M NaCl-0.1M Tris, pH 8.2 before the RNA inoculation.
 b. Individual plaque counts are shown to demonstrate the quality of replication among plates and among dilutions for the various diluents.
 c. TM = too many to count.

2. Conditioning Time

The term "conditioning time" is used here to designate the interval between application of the final wash (1M NaCl-0.1M Tris, pH 8.2) and inoculation of monolayers with the RNA. Figure 1 shows the effect of conditioning time on plaque formation. The monolayers were susceptible to the RNA immediately after the last wash, indicating that preliminary conditioning of the cells with hypertonic salt solution occurred very rapidly. However, higher plaque counts were obtained when the RNA was inoculated from 5 to 13 minutes after application and removal of the last wash. Possible explanations for increase in plaque number could be that time is required both to inhibit tissue ribonuclease^{7,8} and to establish an equilibrium between the osmotic pressure within the cells and that of the medium.⁹

3. Adsorption Time and Temperature

The term "adsorption time" is used here to indicate the interval of time between inoculation with the RNA and removal of the excess inoculum, which was followed immediately by application of the agar overlay. Figure 2 shows the influence of the length of time and the temperature of adsorption on plaque formation by EEE virus infectious RNA. A true plateau was not obtained but a peak was observed after 30 minutes at 25°C. A minor peak was observed when the RNA was adsorbed for 15 minutes at 37°C. The results in Figure 2 clearly show that the highest counts were obtained when the infectious RNA was adsorbed at 25°C for 30 minutes. Since intervals longer than 30 minutes at 25°C seemed to be deleterious for the cells and resulted in a reduced plaque count, the time for adsorption was restricted to more than 25 minutes but less than 30 minutes.

4. Influence of Washing Monolayers

In these experiments, conditions for adsorption, conditioning time, number of cells plated, and diluent for the RNA were those found to be optimal for plaque formation. Before monolayers were inoculated with the infectious RNA, the fluid growth medium was removed and plates were divided into groups to receive eight different wash treatments as indicated in Table III, which shows the results of a typical experiment.

When the cells were given three washes, as in Group 8, the highest plaque counts of the experiment were obtained. There was agreement among dilutions, and results were consistent when a given sample was assayed on different days. Bentonite, a known ribonuclease inhibitor, was tested to determine whether the effect of the washings was due entirely to the elimination of this enzyme. The results showed that in the presence of bentonite the cells still required treatment with hypertonic salt in order to obtain high plaque counts. The effect of washing, therefore, was not simply the elimination of ribonuclease.

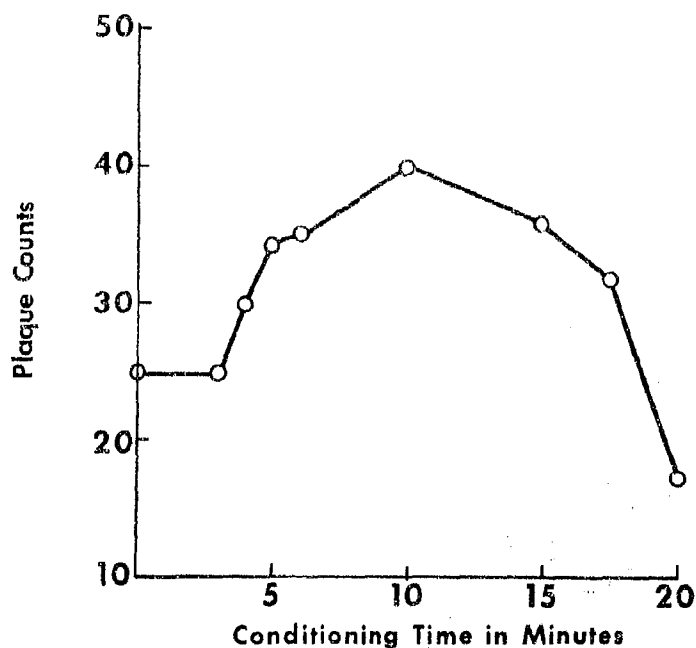


Figure 1. Effect of Conditioning Time on Plaque Formation by EEE Virus Infectious RNA. Each point represents the average of three experiments.

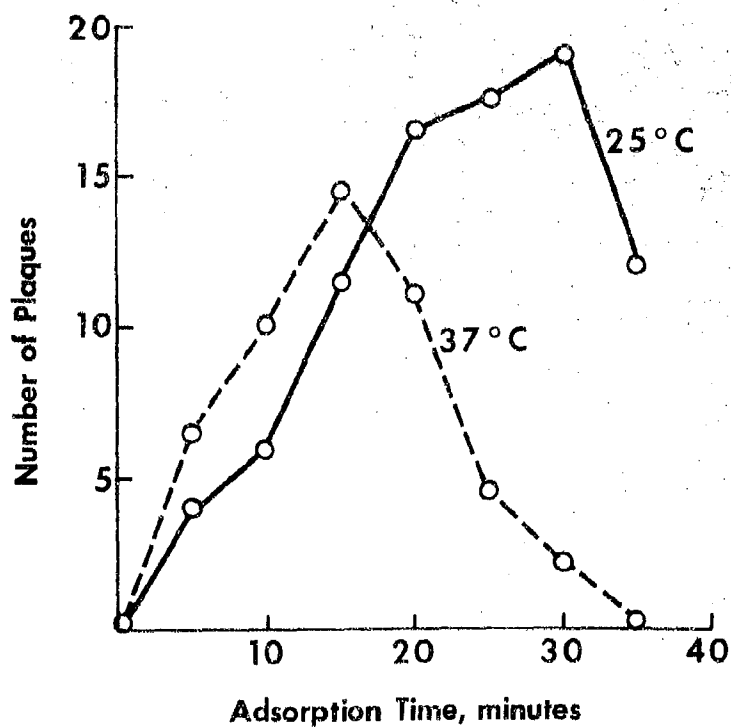


Figure 2. Effect of Adsorption Time and Temperature of Adsorption on Plaque Formation by EEE Virus Infectious RNA. Each point represents the average of three determinations.

TABLE III. PLAQUE FORMATION BY EEE VIRUS RNA:
INFLUENCE OF WASHING MONOLAYERS

GROUP	WASHES	DILUTION OF RNA	PLAQUE COUNTS, per plate	TITER, ^a / pfu/ml
1	None	10 ^{-3.0} 10 ^{-4.0}	2,0 0,0	10 ^{4.0}
2	Saline A ^b /	10 ^{-3.0} 10 ^{-4.0} 10 ^{-5.0}	55,95 7,3 5,0	10 ^{6.0}
3	0.5M NaCl	10 ^{-4.0} 10 ^{-4.7}	44,140,103 40,26,37	10 ^{7.2}
4	1M NaCl, pH 8.2	10 ^{-4.0} 10 ^{-4.7}	180,80 18,19	10 ^{7.0}
5	Saline A, 0.5M NaCl	10 ^{-4.0} 10 ^{-4.7}	140,150 53,30	10 ^{7.3}
6	Saline A, 1M NaCl, pH 8.2	10 ^{-4.0} 10 ^{-4.7}	80,119 12,27	10 ^{7.0}
7	0.5M NaCl, 1M NaCl, pH 8.2	10 ^{-4.0} 10 ^{-4.7}	153,178 50,41	10 ^{7.4}
8	Saline A, 0.5M NaCl, 1M NaCl, pH 8.2	10 ^{-4.7} 10 ⁻⁵	51,85,77 44,55,63	10 ^{7.7}

a. Titer is based on all recorded counts.

b. Saline A: 0.8% NaCl, 0.04% KCl, 0.1% glucose, and 0.002% phenol red in demineralized H₂O brought to pH 7.2 to 7.5 with NaHCO₃.

5. Influence of Original Cell Count

The influence of the number of cells plated on the number of plaques formed was examined using 24-hour monolayers, 1M NaCl-Tris (pH 8.2) as diluent, adsorption time of 25 minutes, and more than five but less than 13 minutes conditioning time. The data in Figure 3 represent average values from four experiments and clearly show the importance of the original cell count on the number of plaques formed. The greatest number of plaques was obtained when 17.5×10^6 to 20×10^6 cells per plate (60 mm by 15 mm) were

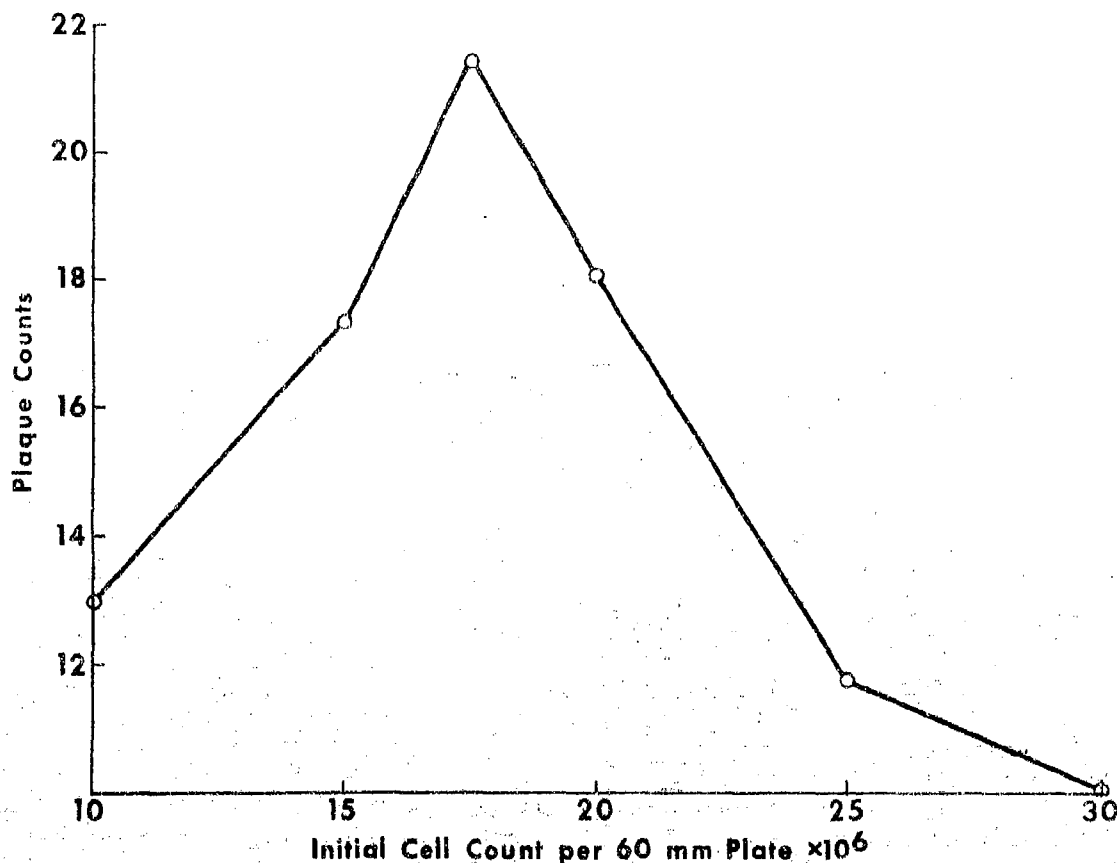


Figure 3. Influence of Original Number of Cells Plated to Form the Monolayers on the Number of Plaques Formed by the Infectious RNA of EEE Virus. Each point represents average values from four experiments.

used. When 15×10^6 cells or less per plate were used, plaques were formed but in addition to being fewer in number, they were small and irregular in appearance; there was also poor replication of the counts and the cells did not stain satisfactorily.

6. Effect of Protamine Sulfate and DEAE-Dextran

Takemori and Nomura¹⁰ showed that minute plaque mutants were inhibited by an extract of agar and that the inhibitory factor (a polysaccharide) was responsible for the failure of these mutants to develop plaques of normal size. Takemoto and Liebhaber¹¹ reported similar findings with encephalomyocarditis virus in which two distinct plaque types were studied. Colón *et al.*^{12,13} found that certain equine encephalitis viruses were also inhibited by the polysaccharide. Plaque formation by virulent VEE virus was unaffected by the inhibitor, but plaque size and number of plaques formed by viruses of EEE, WEE, and attenuated strains of VEE were greatly reduced. The action of the inhibitor was reversed by DEAE-dextran or protamine sulfate (salmine).

A study was undertaken to determine the effect of DEAE-dextran or protamine sulfate on plaque formation by infectious RNA. Although plaque counts were the same in plates with or without DEAE-dextran or protamine sulfate in the overlay, the plaques were three to four times larger in the presence of protamine or DEAE-dextran than in the control plates. We use DEAE-dextran routinely in our assays because it assures development of well-defined plaques and thereby facilitates the counting procedure. One per cent stock solutions of DEAE-dextran (or protamine sulfate - salmine) were prepared in sterile demineralized water and stored at 4°C. For plates to be read 48 hours after inoculation with EEE or WEE infectious RNA (or intact virus), one milliliter of the stock solution was added to the agar portion of 100 milliliters of the overlay medium either before or after sterilization of the agar, giving a concentration of 100 micrograms per milliliter of additive in the final overlay.

7. Effect of Other Factors

When the effect of the age of cell sheets was tested, it was found that the best results were obtained when monolayers were 24 to 48 hours old. Normally, plaque formation requires 48 hours of incubation following inoculation of monolayers with EEE virus or infectious RNA. If 150 micrograms per milliliter of DEAE-dextran or protamine sulfate were included in the overlay and 48-hour monolayers were used for inoculation, plaques of one to two millimeters were obtained with EEE virus or RNA after 24 hours' incubation.

The most convenient inoculum volume was found to be 0.1 milliliter. Tests in which the inoculum was spread over the monolayer varying numbers of times showed no significant difference in the number of plaques formed. This indicated that it was necessary to spread the virus or RNA inoculum carefully over the entire surface of the monolayer only one time early in the adsorption period.

The most consistent results were obtained with the use of plastic Petri dishes (Falcon*). With these plates, no problems were encountered with respect to occasional erratic variation among replicate plates or entire lots of poor monolayers. Both of these problems were experienced with glass dishes.

* Source: Falcon Plastics, Division of B-D Laboratories, Inc., 5500 W. 83rd St., Los Angeles, California, (TCPD-6015, #3002).

IV. STANDARD PROCEDURE FOR ASSAY OF INFECTIOUS RNA FROM THE EQUINE ENCEPHALITIS VIRUSES

A. PROCEDURE

Based on the above results, the following procedure for plaque titration of the RNA of EEE virus was finally adopted. RNA was diluted in 1M NaCl-0.1M Tris-HCl buffer, pH 8.2. A volume of 0.1 milliliter of the diluted RNA was plated onto chick fibroblast monolayers that had been prepared with an initial cell count of 20×10^6 cells per 60-mm plastic Petri dish and grown for 24 to 48 hours. The monolayers were washed with saline A, then with 0.5M NaCl, and finally with RNA diluent (1M NaCl-Tris-HCl buffer, pH 8.2) using two milliliters of each per plate. After a conditioning time of 5-13 minutes, each monolayer was inoculated with 0.1 milliliter of the desired dilution of RNA. The inoculum was thoroughly spread one time and the plates were incubated at 25°C for 25 minutes; the excess inoculum was removed and the plates were overlaid with nutrient agar prepared as described in Section II. The overlay included 100 micrograms of DEAE-dextran per milliliter of overlay if the plates were to be read 48 hours after inoculation with RNA, or 150 micrograms of DEAE-dextran per milliliter of overlay was used if the plates were to be read after 24 hours. The plates were incubated at 37°C in a humidified atmosphere of five per cent carbon dioxide and 95 per cent air. The plates were stained with neutral red (two milliliters per plate of 1:8000 aqueous solution). This procedure was also used with the same efficiency for the infectious RNA of VEE and WEE viruses.

B. REPRODUCIBILITY OF RNA ASSAY SYSTEM

The results of nine assays on different dates of a preparation of EEE virus infectious RNA using the procedure just described are shown in Table IV. Also shown are the results of nine assays, on different dates, of intact EEE virus. These data demonstrate that the assay of RNA infectivity is as reliable as that for the intact virus. The assay system as described here for the infectious RNA (omitting the use of hypertonic salt solutions), when used for the assay of the infectivity of intact virus, has shown more sensitivity, reliability, and reproducibility than the methods previously used for the assay of the equine encephalitis viruses.

Repeated assays by this procedure revealed a strictly linear dose-response relationship between the dilution of the inoculum and the number of plaques, as shown in Figure 4. This observation is similar to that made by Parker¹⁴ with vaccinia and myxoma viruses, by Ellis and Delbrück¹⁵ with bacteriophage, by Bachrach¹⁶ with the RNA of foot-and-mouth disease virus, and by Alexander *et al.*,¹⁷ Koch *et al.*,¹⁸ and Holland *et al.*¹⁸ with the RNA from poliovirus. It suggests that only a single element is required to infect.

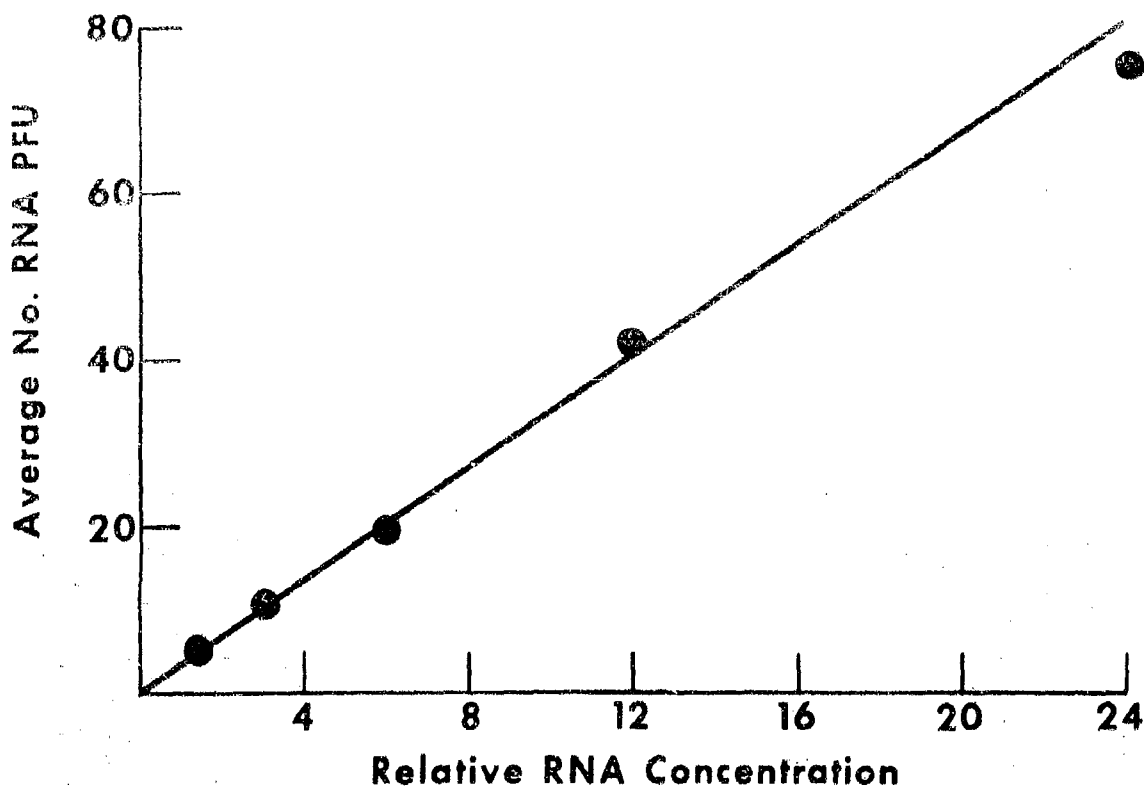


Figure 4. Dose-Response Relationship for EEE Virus Infectious RNA in 1M NaCl, pH 8.2, as Diluent on Chick Fibroblast Monolayers. Each point represents the average of four experiments.

Several experiments were performed to determine whether the frequency distribution of plaques from a suspension of infectious RNA from EEE virus was of the Poisson type; such a similarity has been noted with RNA of foot-and-mouth disease virus by Bachrach¹⁸ and by Koch *et al*⁶ with RNA of poliovirus. In each experiment, a series of plates was inoculated with equal volumes of an RNA suspension of low concentration. The distribution of plaques was found to agree with that expected from the Poisson equation, as shown in Table V. The close agreement between calculated and experimental values, repeated in all experiments, represents direct evidence that the number of plaques does represent the number of independently distributed RNA units.

TABLE IV. REPRODUCIBILITY OF TITERS OF EEE INTACT VIRUS AND OF EEE INFECTIOUS RNA BY THE PLAQUE ASSAY SYSTEM

ASSAYS OF EEE INTACT VIRUS ^{a/}		ASSAYS OF EEE INFECTIOUS RNA ^{b/}	
Trial No.	10 ⁹ pfu/ml	Trial No.	10 ⁷ pfu/ml
1	1.2	1	2.5
2	1.6	2	3.1
3	1.5	3	3.0
4	1.2	4	2.5
5	1.1	5	2.7
6	1.1	6	3.1
7	1.1	7	3.3
8	1.2	8	2.5
9	1.3	9	3.1
Average	1.3	Average	2.9

- a. Intact virus assays were performed on frozen samples of a 10⁻² dilution of tissue culture supernate of infected fibroblast monolayers. The results cover a three-month period and are listed in chronological order of assays.
- b. Infectious RNA was prepared from chick embryos infected with EEE virus and was stored at -65°C. There was a period of 10 months between the first and last trial and results are listed in chronological order of assays.

TABLE V. PLAQUE FORMATION^{a/} BY EEE VIRUS RNA: FREQUENCY DISTRIBUTION

SAMPLE	P _r (Experimental)	P _r (Calculated) ^{b/}
0 plaques on 12 plates	0.387	0.393
1 plaque on 12 plates	0.387	0.369
2 plaques on 5 plates	0.161	0.172
3 plaques on 1 plate	0.032	0.053
4 plaques on 1 plate	<u>0.032</u>	<u>0.013</u>
	0.999	1.000

- a. Thirty-one 0.1-milliliter samples of a dilute suspension of infectious RNA from EEE virus were plated for plaque counts.
- b. The calculated distribution corresponds to the equation:

$$P_r = \frac{s^r e^{-s}}{r!}, \text{ where } s = \text{average number of particles per sample,}$$

r = the actual number in a given sample,
and

p_r = the probability of having r particles
in a given sample.

V. DISCUSSION

The factors that affect plaque formation by the infectious RNA of the equine encephalitis viruses may be separated into two groups: factors that affect the RNA molecule and those that affect the host cell. This was found to be true for plaque formation by the RNA of poliovirus.¹⁹ The RNA molecule is affected by metals, enzymes, salt concentration, etc. The physiological state of the host cell at the time of exposure to RNA is influenced by the age of the cells, thickness of monolayers, temperature and time of adsorption, molarity of the solutions, and other unknown factors. Some factors, like the hypertonic solutions, affect both the configuration of the RNA molecule¹⁰ and the physiological state of the host cell. In our laboratory we have found that when precautions are taken to control the known influential factors, measurement of RNA infectivity by plaque assay is as reproducible as that for whole virus.

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